

## REPORT DOCUMENTATION PAGE

Form Approved  
OMB No. 0704-0188

The public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing the burden, to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.

PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. REPORT DATE (DD-MM-YYYY) 15-07-2004		2. REPORT TYPE Final Report		3. DATES COVERED (From - To) 01 March 2001 - 15 July 2004	
4. TITLE AND SUBTITLE Neuroprotective Effects of Opioid-Like Hibernation Factors in Cerebral Ischemia				5a. CONTRACT NUMBER _____	
				5b. GRANT NUMBER N00014-01-0494	
				5c. PROGRAM ELEMENT NUMBER _____	
6. AUTHOR(S) Oeltgen, Peter, R.				5d. PROJECT NUMBER _____	
				5e. TASK NUMBER _____	
				5f. WORK UNIT NUMBER _____	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Kentucky Research Foundation 109 Kinkead Hall Lexington, KY 40506-0057				8. PERFORMING ORGANIZATION REPORT NUMBER _____	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Office of Naval Research 800 N. Quincy Street Arlington, VA 22217-5000				10. SPONSOR/MONITOR'S ACRONYM(S) ONR	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S) _____	
12. DISTRIBUTION/AVAILABILITY STATEMENT Distribution Unlimited					
13. SUPPLEMENTARY NOTES _____					
<b>DISTRIBUTION STATEMENT A</b> Approved for Public Release Distribution Unlimited					
14. ABSTRACT We have shown that infusions of opioid-like hibernation factors can provide ischemic protection in vivo and in vitro. In a mouse cerebral ischemia model, we showed a marked reduction in cerebral infarct volume and behavioral deficits when Deltorphins-A, D and Dermorphin-H were infused 1 hr after middle cerebral artery occlusion. Using mice deficient in either neuronal (nNOS), inducible (iNOS) or endothelial (eNOS) nitric oxide synthase, we showed that only endothelial NOS plays a key role in cerebral ischemic protection. We found that Deltorphin-D inhibited nitric oxide (NO) release in a dose dependent manner when mouse N9 microglial cells were activated by lipopolysaccharide (LPS) and interferon-gamma, giving further evidence that the neuroprotective effect of these delta opioids may include their ability to retard or block the release of NO and reactive oxygen species which occur in ischemic processes such as hemorrhagic shock, myocardial infarction, and stroke. Most recently, we showed that Deltorphin-D increased blood pressure and enhances 6 hr survival of rats undergoing hemorrhagic shock without concomitant fluid resuscitation.					
15. SUBJECT TERMS Opioids, Deltorphins, Dermorphins, Ischemic injury					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT  UU	18. NUMBER OF PAGES  5	19a. NAME OF RESPONSIBLE PERSON Peter R. Oeltgen, Ph.D
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (Include area code) (859) 381-5939

Standard Form 298 (Rev. 8/98)  
Prescribed by ANSI Std. Z39.18

20040722 070

## **FINAL REPORT**

**GRANT #:** N00014-01-1-0494

**INSTITUTION:** University of Kentucky College of Medicine

**GRANT TITLE:** Neuroprotective Effects of Opioid-Like Hibernation Factors in Cerebral Ischemia

**AWARD PERIOD:** 1 March 2001- 31 March 2004

**OBJECTIVE:** To determine the mechanism(s) by which opioid-like hibernation factors provide cerebral ischemic protection and increase blood pressure and survival following hemorrhage shock in clinically relevant animal models.

### **APPROACH:**

1. **Cerebral Ischemia Studies:** To determine if nitric oxide (NO) mediates the neuroprotective effects of highly specific delta opioids (Deltorphin A, Deltorphin-D and Demorphin-H). Wild type and nitric oxide deficient (NOS) all on a C57B16 background were subjected to one hr occlusion of the middle cerebral artery (MCA) and 24 hr of reperfusion. Brain infarct volume and behavioral deficits were measured in control mice and in mice which were deficient in inducible nitric oxide (iNOS), neuronal nitric oxide (nNOS) endothelial cell nitric oxide (eNOS).

### **ACCOMPLISHMENTS:**

Injection of saline into the NOS deficient mice resulted in infarct volumes previously demonstrated in the literature, where iNOS and nNOS deficient animals have reduced damage due to the detrimental effects of NO synthase in the brain. In contrast deficient animals showed an increase in infarct volume indicating a protective effect of eNOS in vascular function in cerebral ischemia. Injection of the Deltorphin-A, Deltorphin-D and Dermorphin-H resulted in a significant protection of the brain in wild-type animals and infusion gave rise to similar effects in the NOS deficient mice as seen in the saline injected study. Since there was no enhancement of protection in the NOS deficient mice, these data suggest that some of the protective effects of Deltorphin-D are mediated through nitric oxide.

### **CONCLUSIONS:**

Our data indicate that opioids, which are highly specific for the  $\delta_2$  opioid receptor subtype, can provide profound cerebral ischemia protection (i.e., significantly decreased infarct volume vs. controls) when infused in the tail vein of mice one hour after occlusion of the middle cerebral artery. Utilizing gene inactivation mice indicates that  $\delta$  opioid ischemic neuro protection may involve a mechanism requiring activation of endothelial cell NO synthase.

**DISTRIBUTION STATEMENT A**  
Approved for Public Release  
Distribution Unlimited

## **APPROACH:**

### **2. In Vitro Cell Culture Assay To Monitor The Nitric Oxide (NO) Inhibitory Activity of Delta Opioid-Like Hibernation Factors.**

To develop a rapid *in vitro* cell culture assay by which we could monitor NO inhibitory activity of *delta* opioid-like hibernation factors alone or in combination with curcumin, a potent antioxidant. This was accomplished utilizing the Griess Assay (Oshima et al, Carcinogenesis 12: 1217-1220, 1991)

#### **ACCOMPLISHMENTS:**

Four different opioid peptides were used to test their effect on inhibition of NO production in an *in-vitro* cell culture assay. Delt-D, DPDPE (D-Pen<sup>2,5</sup>)-Enkephalin, a *delta*<sub>1</sub> selective opioid. DADLE (D-Ala<sup>2</sup>-Leu<sup>5</sup>-Enkephalin), a non-specific *delta* opioid and Dermorphin-H a *mu* selective opioid were used. In addition Curcumin, an antioxidant reactive oxygen species (ROS) scavenger and known NO synthase inhibitor was also used as a positive control. Among the opioids only Delt-D inhibited NO production in a concentration-dependent manner. Delt-D at a concentration of 1 mM inhibited NO production by 95% when compared to activated control cells. Curcumin at a concentration of 50  $\mu$ M also inhibited NO production by 95% when compared to activated control cells whereas even 2.5 mM concentration, DADLE has no effect on NO release by activated cells. In activated cells that were treated with 1 mM DPDPE there was a slight increase in NO production when compared to activated control cells.

#### **CONCLUSIONS:**

Our data indicates that only Delt-D, a *delta* opioid which is highly specific for the *delta*<sub>2</sub> receptor subtype, inhibits NO release in an activated microglial cell line in a concentration dependent manner. DPDPE, which is a *delta* opioid highly specific for the *delta*<sub>1</sub> receptor subtype does not inhibit NO release but in fact enhances NO release compared to controls. These data indicated that opioids binding the *delta*<sub>2</sub> receptor subtype may initiate cellular mechanisms resulting in ischemic protection.

## **APPROACH:**

**3. Hemorrhagic Shock Studies:** Rats weighing 300-350 g had catheters placed in the femoral artery (for hemorrhage), tail artery for blood pressure (BP) measurement and the tail vein (for administration of opioids) controls received saline or opioids without hemorrhage. BP and 6 hr survival were monitored.

**Moderate Hemorrhage Protocol:** For the moderate hemorrhage studies (5.5 ml hemorrhage volume prior animals received saline or Delt-D to hemorrhage without fluid resuscitation and post-treated animals received saline or Delt-D 2 mg/kg following hemorrhage without fluid resuscitation. BP, blood loss and rectal temp, at beginning and end of hemorrhage were determined. The effect of Delt-D infusions on the expression of Ubiquitin B and C (UBB and UBC) was determined. Heat Shock Protein (HSP-70), and inducible Nitric Oxide Synthase (iNOS) mRNA transcripts in heart, leg and brain were determined after 2 hr.

**ACCOMPLISHMENTS: Moderate Hemorrhage Protocol:** Preinfusions of Delt-D did not significantly effect BP while 2 mg/kg post hemorrhage infusions without

resuscitation fluid significantly increased BP compared to controls and decreased core temp by 4.5 °F compared to controls. Delt-D infusions increased iNOS and HSP70 mRNA in heart and leg in non-hemorrhaged controls and UBB in brain of non-hemorrhaged controls. Pre-treated Delt-D animals had elevated brain iNOS and HSP70 mRNA and post-hemorrhage Delt-D treated animals had elevated UBC mRNA in heart and brain and HSP70 mRNA in leg tissue.

#### **APPROACH:**

**Severe Hemorrhage Protocol:** For the severe hemorrhage protocol (9.0 – 11.0 ml hemorrhage volume representing 53-61% of total blood volume), rats were infused with either 3.0 mg/kg of a highly specific *mu* opioid ZGI-06, (n=11), in 1.0ml PBS, or a Delt-D variant (ZGI-07 n=11) and ischemic tolerance (ie BP and 6 hr survival) was monitored. Controls (n=6) were infused with 1.0 ml PBS.

#### **ACCOMPLISHMENTS:**

**Severe Hemorrhage Protocol:** Six hr survival was 33% for controls (n=2), 60% for ZGI-06 (n=6) and 72% for ZGI-07 (n=8) BP increased within 30-45 seconds after infusion of ZGI-06 by  $29.5 \pm 13.0$  mmHg vs. control ( $p=0.01$ ) and  $38.8 \pm 18.5$  mmHg for ZGI-07 vs. control, ( $p=0.002$ ).

#### **CONCLUSIONS:**

##### **Moderate Hemorrhage Protocol:**

Pre-hemorrhage infusions of Deltorphan-D do not significantly alter BP compared to saline controls. Delt-D at 2 mg/kg increase blood pressure and decreased core temperature vs. saline controls during the 1<sup>st</sup> hour of hemorrhage without concomitant fluid resuscitation.

##### **Severe Hemorrhage Protocol:**

Highly specific *delta* opioid(ZGI-07) and a highly specific *mu* opioid (ZGI-06) increased BP and enhanced 6 hr. survival in rat undergoing profound hemorrhage (50% blood loss or>) without concomitant fluid resuscitation.

#### **PUBLICATIONS:**

##### **PUBLICATIONS AND ABSTRACTS FOR TOTAL GRANT PERIOD.**

Sigg, D., Coles, J.A., Gallagher, W. J., OELTGEN, P.R., and Iazzo, P.A. Opioid Cardioprotection, Myocardial Function and Energy Metabolism. *Annals of Thoracic Surgery*, 72: 1576-1582, 2001.

Bolling, S. F., Badhwar, V., Schwartz, D.F., OELTGEN, P.R., Kilgore, K., Su, T-P. Opioids Confer Myocardial Tolerance to Ischemia: Interaction of Delta Opioids Agonists and Antagonists. *J. Thorac and Cardiovas. Surgery* 122:476-4781, 2001.

Karck, M., Tanaka, S., Bolling, S.F., Simon, A., Su, T-P, OELTGEN, P.R., and Haverich, A. Myocardial Protection by Ischemic Preconditioning and  $\delta$ -Opioid Receptor

Activation in the Isolated Working Rat Heart. *J. Thoracic & Cardiovasc. Surg.* 122: 986-992, 2001.

Gottsch, H., Thomas, D., **OELTGEN, P.R.** AND Smith-Sonneborn, J. Ubiquitin Stress Response in Rat Heart Treated With Hibernating Serum, *Gerontological Society of American 54<sup>th</sup> Annual Meeting*, Chicago, IL. Nov. 14-18, 2001.

Rudich, S.M., Arenas, J.D., **OELTGEN, P.R.**, AND Chang, W-J. Can Hibernation Factors Protect the Rodent Liver From Cold Storage/Reperfusion Injury. *American Transplant Congress*, Miami, FL. Abstract #251656, April 30-May 2, 2002.

Sigg, D., Coles, J.A., **OELTGEN, P.R.**, and Iazzo, P.A. Preconditioning With Delta-Opioid Receptor Agonists Decreases Myocardial Infarct Size, But not Sublethal Arrhythmias in Swine. *Experimental Biology* 2002, New Orleans, LA. April 20-24, 2002. Abstract #402.5, P.A488, FASEB. J. 16(4), 2002

Govindaswami, M., Rodgers, J.R., Lesnaw, J.A., **OELTGEN, P.R.** A Cell Culture Assay for Delta Opioids and Opioid-Like Hibernation Specific Factors (HSF). *Experimental Biology* 2002, New Orleans, LA. April 20-24, 2002. Abstract # 643.25.

Govindaswami, M., Bishop, P.D., Kindy, M.S., **OELTGEN, P.R.** Neuroprotective Effects of Opioid-Like Hibernation Factors in Cerebral Ischemia. *Experimental Biology* 2003, San Diego, CA. April 11-15, 2003. Abstract #579, 10P.A895 FASEB.J. 17.

McBride, S.M., Smith-Sonneborn, J., **OELTGEN, P.R.**, Newton, S.V., Flynn, F.W., Modulation of Hemorrhagic Shock by Deltorphin-D, A delta2 opioid receptor agonist. *Experimental Biology* 2003. San Diego, CA. April 11-15, 2003. Abstract #806, 12P.

Smith-Sonneborn, J., Gottsch, H., Cubin, E., Thomas, P. and **OELTGEN, P.R.** Strategy for Stress Tolerance: Opioids. *J. Gerontology. Biological Sciences* 59A, (5) 433-440, 2004.

#### **PATIENTS ISSUED:**

United States Patent No. 6,316,411 B1. **OELTGEN et al.** Protection Against Ischemia and Reperfusion Injury. Issued Nov. 13, 2001.

United States Patent No. US 6,380,164 B1. **OELTGEN et al.** Method for Treating Cytokine Mediated Hepatic Injury. Issued April 30, 2002.

United States Patent No. 6,544,950. **OELTGEN AND Kindy**, Seventeen Amino Acid Peptide (Peptide-P) for Treating Ischemia and Reperfusion Injury. Issued April 3, 2002.